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Prothrombin Mutant, Factor V Leiden, and Thermolabile Variant of Methylenetetrahidrofolate Reductase Among Patients With Sickle Cell Disease in Brazil

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The prevalence of the prothrombin gene variant (allele 20.210 A), factor V Leiden mutation, and homozygosity for transition 677C→T in the methylenetetrahydrofolate reductase (MTHFR) gene was determined among patients with sickle cell disease (SCD). The group included 73 patients with median age of 32.3 years with a diagnosis of sickle cell anemia in 53 patients, hemoglobinopathy SC in 16 patients, and four with S/β⁰ thalassemia. Vascular complications such as ischemic stroke or deep vein thrombosis were diagnosed in nine patients. Heterozygosity for the prothrombin gene variant or factor V Leiden mutation was identified in four patients. However, only one patient, who developed ischemic stroke, was identified as a carrier of factor V Leiden mutation. None of the patients presented homozygosity for the thermolabile variant of the MTHFR. These data suggest a low clinical impact of inherited hypercoagulability risk factors in developing thrombosis, occlusive stroke, or mortality data among patients with SCD in Brazil. Am. J. Hematol. 59:46–50, 1998. © 1998 Wiley-Liss, Inc.

Key words: factor V gene; prothrombin gene; homocysteine; sickle cell disease; thrombosis

INTRODUCTION

Vascular thrombosis is a common complication in patients with sickle cell disease (SCD), and coagulation and fibrinolysis abnormalities leading to the development of hypercoagulability have often been described in these patients [1–8]. Among adult patients with SCD, acute chest syndrome as well as occlusive strokes, were identified as major cause of death [9–11], and these complications resulted from previous thromboembolism events and endothelial cell damage [11–13]. The recognition of individuals with a genetically determined high risk for developing vascular disease among those with SCD would be useful, particularly for a potential primary prevention of occlusive disorders by means of antithrombotic therapy [3,14–15].

In the general population, a hereditary tendency for venous thrombosis, known as inherited thrombophilia, has been associated in 20–50% of the cases with a poor anti-coagulant response of plasma to activated protein C [16–19]. The molecular basis of this mechanism is a 1691G→A transition in the clotting factor V gene resulting in a Arg506→Gln (factor V Leiden) substitution [17]. The second most common risk factor for vascular thrombosis was recently described by Poort et al. [20] and results from a transition 20.210 G→A in the 3′-untranslated region of the prothrombin gene and was associated with higher prothrombin levels and with a threefold greater risk of venous thrombosis. Arterial thrombosis is rarely associated with the causes of inherited thrombophilias [21,22], but is usually related to mild hyperhomocysteinemia [23,24]. Among individuals with inherited hyperhomocysteinemia, those homozygous for the thermolabile variant of the methylenetetrahydrofolate reductase (MTHFR-T) [25], resulting from a 677C→T substitution which produces an Ala677→Val switch, have elevated plasma homocysteine levels; this elevation

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represents an important genetic risk factor for vascular disease [26]. We have previously described that being homozygous for the mutated allele MTHFR-T increases the risk for arterial disease by fivefold and for venous thrombosis by threefold [27]. Moreover, the homozygous for MTFR-T, and heterozygous for factor V Leiden or prothrombin gene variant were found in 4%, 2%, and 1% of the general Brazilian population, respectively [27–29], and a possible role of these genetic factors in the vascular complication of SCD could be considered. In this study, we have determined the prevalence and clinical impact of factor V Leiden, the prothrombin gene variant (nt20210A), and the homozygosity for MTHFR-T among adult patients with SCD.

PATIENTS AND METHODS

Selection of Patients

The study included patients at the Hemoglobinopathy outpatient clinics of the University Hospital of the State University of Campinas, followed during the last 10 years. The clinical data and blood samples were collected after patient's or parent's consent during regular visits. The group included 73 patients (40 female and 33 males), median age of 32.3 years (range, 14 to 62 years) with a diagnosis of sickle cell anemia (SCD) in 53 patients, hemoglobinopathy SC in 16 patients, and 4 with S/β^0 thalassemia. In 16 out of 54 patients, a diagnosis of α-thalassemia was characterized. The β^s haplotype was Ben/Car in 30 out of 53 patients with SCA, followed by 15 homozygous for Ben and 14 homozygous for Car haplotype. Among patients with hemoglobinopathy SC, the β^C haplotype, Ben/I was identified in eight patients, Car/I in three patients, Car/II in two patients, and one patient with Sen/I, and a Car/atypical haplotype was identified in another.

Laboratory Methods

The diagnosis of SCD was confirmed by hemoglobin electrophoresis performed on acetate cellulose membrane, pH 8.6; and citrate agar gel, pH 6.2; and in most cases, was confirmed by family studies. Hemoglobin (Hb) A2 were eluted for quantification, Hb F concentrations were determined as described previously, and the globin gene haplotypes were determined as described elsewhere [30]. Genomic DNA obtained from peripheral blood of the patients and the controls was extracted by a standard method [31].

Detection of Mutation Arg506→Gln in the Factor V Gene (Factor V Leiden)

This mutation was identified as described previously by amplification of exon 10 of the factor V gene using the polymerase chain reaction (PCR) with 5′-CTTGAAGGAAATGCCCCATTA-3′ as the primer sense and 5′-TGCCCAGTGCTTAACAAGACCA-3′ as the antisense primer followed by further digestion of the PCR product with endonuclease Mnl I [28].

Diagnosis of the Mutation Ala677→Val in the MTHFR Gene

A fragment of the MTHFR gene was amplified by PCR using the primers (described by Froost et al. [26]) sense 5′-TGAAGGAGAAGGTGTCTGCGGGA-3′ and antisense 5′-AGGACGGTGCGGTGAGAGTG-3′. The PCR product was digested with endonuclease Hinf I as previously published.

Diagnosis of Mutation 202021G→A in the Prothrombin Gene

A fragment of the 3′-untranslated region of the prothrombin gene was amplified by PCR in a mixture of 54 mM Tris-HCl, pH 8.8; 5.4 mM $MgCl_2$; 5.4 μM 0.8 mM of each nucleoside triphosphate; 400 ng of each primer described by Poort et al. [20], sense 5′-TCTAGAAACAGTTGCCTGGC-3′ and mutagenic antisense 5′-ATAGCACTGGGAGCATTGAAGC-3′; 500 ng of genomic DNA; and two U of Taq polymerase. The reaction involved 35 cycles of incubation at 94°C (one min), 58°C (one min), and 72°C (one min). A fragment of 345 bp was obtained and 10–15 μl of the PCR product were digested overnight using 2.5 U of endonuclease Hind III. Following digestion of the mutated prothrombin gene (allele 20210A), a fragment of 322 bp was observed in a 2% agarose gel. When the normal allele 20210G was present, there was no cleavage site for Hind III and the 345 bp fragment remained intact (Fig. 1).

RESULTS

Thromboembolic Complications

Two patients had an objective diagnosis of venous thrombosis: one patient presented with a spontaneous episode in the lower extremity, and the other patient had a severe congestive cardiac failure at the time thrombosis was diagnosed. One patient developed a thrombosis associated with central vein catheter; the diagnosis was based on clinical data.

Vascular occlusive cerebral disease was diagnosed in five patients based on focal neurologic deficit and computerized tomography in all cases, and arteriography in two out of five cases. One patient with hemoglobinopathy SC presented with a transitory cerebral ischemic event (see Table I).

Chronic Complications

Priapism occurred at a post-puberal age in five out of 33 male patients (15%) and all five males presented with SCA.

Leg ulcers were present in 18 patients (eight females,

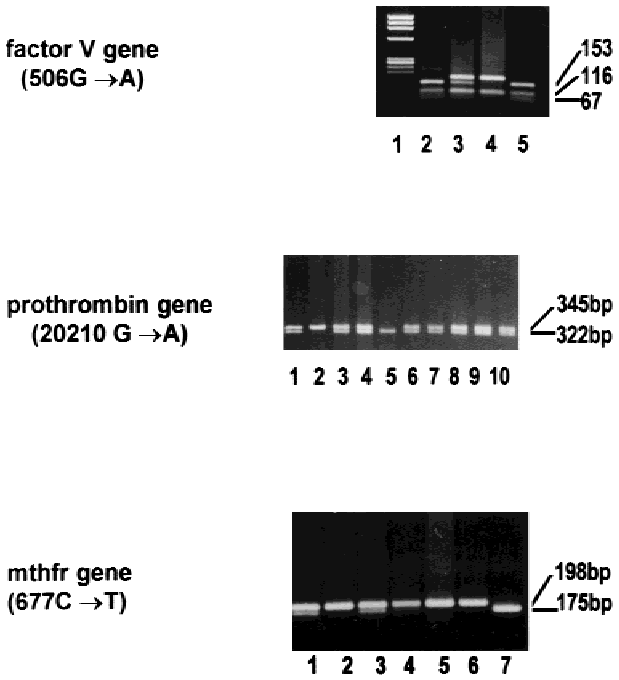

Fig. 1. Ethidium bromide-stained 2% agarose gel showing PCR products corresponding to a fragment of the factor V gene (top) digested by the Mnl I, the 506G→A transition result in a fragment of the 153 bp as indicated, in line 4 a homozygous for A/A, in line 3 a heterozygous G/A, in lines 2 and 5 normal individuals G/G, and in line 1 molecular weight marker. The prothrombin gene variant (middle) was identified after digestion with HindIII. In the normal individual, as shown in line 2, a fragment of 345 bp is observed 20210 G/G. In line 5, a homozygous for the mutated allele A/A is shown, and in the lines 1, 3–4, and 6–10, heterozygous G/A are observed. For the MTHFR gene alleles (bottom), a digestion with Hinf I results in a normal fragment of 198 bp but when the mutation is present, a fragment of 175 bp results. In line 7 is shown a homozygous for the mutated allele 677 T/T, and in lines 1 and 3, heterozygous C/T.

TABLE I. Prevalence of Inherited Risk Factor for Vascular Disease in Patients With SCD*

	N	Factor V Leiden	Prothrombin variant	Homozygous MTHFR-T
Stroke	5	1	0	0
DVT	3	0	0	0
Leg ulcer	18	0	0	0
Avascular bone necrosis	9	0	1	0
Priapism	5	0	0	0
Overall SCD	73	2	2	0
Black control	137	1	3	2

*SCD, sickle cell disease; MTHFR-T, methylenetetrahydrofolate reductase; DVT, deep venous thrombosis.

10 males), 16 of them presenting with SCA; avascular bone necrosis was diagnosed in nine cases (five female, four males), seven of them with hemoglobinopathy SC, and two with SCA.

Mortality

During the follow-up period, eight of 73 patients died; ages ranged from 20 to 35 years. The circumstances of the deaths were identified as follows: Acute chest syndrome caused by pulmonary emboli confirmed by postmortem findings (one case); pulmonary infection (one case); severe congestive cardiac failure (two cases); systemic infection and hepatic sickle crisis (one case); hemorrhagic stroke (one case); and 2 cases due to unknown cause.

Laboratorial Results

The results revealed the factor V gene mutation in two patients, but heterozygosity for the mutated allele of the prothrombin gene (nt 20210A) was present in another two patients. All but one patient were assymptomatic for vascular disease. A girl with SCA who had an extensive cerebral ischemic event at the age of 13 was identified as heterozygous for the factor V Leiden. She had a sister with SCA who also developed an occlusive stroke which recurred after two years, but this patient did not carry the factor V Leiden mutation. The remaining patients with deep venous thrombosis or ischemic cerebral event presented no inherited risk factor. None of the patients with SCD presented homozygosity for the MTHFR-T.

In the group with chronic complications, one patient with hemoglobinopathy SC was heterozygous for the prothrombin variant and had avascular hip necrosis. Among those patients who died during this period, no risk factors for vascular disease were identified.

The father of an SCA patient heterozygous for the factor V Leiden developed a superficial thrombophlebite and a spontaneous venous thrombosis by the age of 56. Laboratory investigation of the father revealed heterozygosity for the factor V Leiden mutation, and normal plasma levels of protein C, protein S, and antithrombin III.

Comparison of the prevalence of factor V Leiden in SCD in the Brazilian Black population reported previously was distinct but not significant (2.7% vs. 0.7%; $P = 0.27$); this was also observed for the prothrombin variant (2.7% vs. 2%), and homozygosity for the MTHR-T (1% vs. 1.45%; $P = 0.42$).

DISCUSSION

A hypercoagulable state in SCD is well documented based on evidence for increased thrombin generation during pain crisis as well as during a steady state [1–3]. The two major causes of death among adults with SCD are related to vascular occlusion, which suggests that pharmacological manipulation of hemostasis could be useful in treating SCD complications [9,11]. Nevertheless, the hypercoagulability usually results from an acquired condition such as an increase of procoagulant fac-

tors and a decrease of anticoagulant proteins [1–7]. In addition, Wright et al. [6] also described a distinct mechanism of acquired activated protein C resistance among SCA patients, probably resulting from an increased activity of factor VIII:C, which was described among women during therapy with oral contraceptives or pregnancy [32,33].

Inherited risk factors for vascular disease, including venous as well as arterial thrombosis, have been described in the worldwide population [18,19]. Reported among these risk factors was a lower prevalence of the factor V Leiden mutation and homozygosity for the MTHFR-T in individuals of African descent [34–36]. However, the novel risk factor resulting from the prothrombin gene variant was distributed similarly among those of African descent as well as those of Caucasian descent [29]. The ethnical background of the Brazilian population is highly heterogeneous, comprising Caucasians, Africans, and also Indigenous individuals in a great admixture of races. Previously we reported that heterozygosity for factor V Leiden, prothrombin variant, and homozygosity for MTHFR-T were common among a non-select population of Brazil and that the prevalence increased among those with vascular disease [27–29].

In this study, when we analyzed mortality data, development of thrombosis and occlusive stroke, no distinct prevalence of the risk factors was found. In one family, two sisters presented occlusive stroke but only one was a carrier of the factor V Leiden mutation. This is in agreement with the lack of correlation between factor V Leiden mutation and cerebral ischemia among patients with SCD [37], as well as in the general population [38–40].

Stroke in SCD has been associated with high homocysteine levels and inversely correlated to folate levels [41]. Although we did not measure the homocysteine levels, our data were restricted to the homozygous for MTHFR-T, which was associated with a twofold increase in homocysteine plasma levels. However, any SCD patient with or without stroke carried the MTHFR-T in the homozygous state. These data were in accordance with the very low prevalence of MTHFR-T allele among those of African descent [35].

The presence of chronic complications such as priapism, leg ulcers, and avascular bone necrosis was not related to the presence of risk factors studied.

An interesting point is that the latter complication has been related to inherited hypercoagulability among adults with idiopathic osteonecrosis and children with Legg-Calvé−Perthes disease [42,43].

Moreover, the prevalence of the risk factors analyzed was not significantly distinct between the overall group with SCD and the control Black population from Brazil. These data suggest that the search for inherited hypercoagulability did not allow the identification of high-risk patients for occlusive vascular complication of SCD.

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